

Final Report

Study Title:

Acute toxicity test in *Oryzias latipes*

Author:

Osamu Saika

Study Completed on:

July 21, 2010

Test Facility:

Nisso Chemical Analysis Service Co., Ltd. (NCAS)

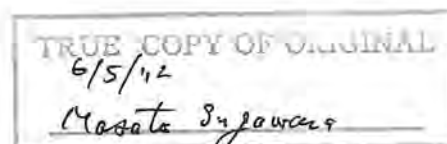
Odawara Laboratory

345 Takada, Odawara, Kanagawa 250-0216, Japan

Sponsor:

Study Number:

NCAS 10-066



GLP Compliance Statement

Study No.: NCAS 10-066

Study title: Acute toxicity test in *Oryzias latipes*

This study was carried out in accordance with the following good laboratory practice regulation;

Standard for the test facility conducting tests concerning new chemical substances, etc. (Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, No. 1121003, November 21, 2003; Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, No.3, November 17, 2003; Environmental Policy Bureau, Ministry of the Environment, No. 031121004) The latest amendment: July 4, 2008.

The final report was prepared faithfully and consistently with the raw data obtained.

Study Director: (signature) (seal) (July 21, 2010)
Osamu Saika
Nisso Chemical Analysis Service Co., Ltd.
Odawara Laboratory

The original signature page of GLP Compliance Statement follows on page 3.

The original GLP Compliance Statement

The English translation of GLP Compliance Statement appears on page 2.

GLP 適合陳述書

試験番号： NCAS 10-066

試験名：

ヒメダカに対する急性毒性試験

この試験は「新規化学物質等に係る試験を実施する試験施設に関する基準について」平成 15 年 11 月 21 日薬食発第 1121003 号、平成 15・11・17 製局第 3 号、環保企発第 031121004 号（最終改正 平成 20 年 7 月 4 日）に従って実施した。

この試験はこの最終報告書に述べられた方法により行われ、この最終報告書は試験実施により得られた生データを正確に反映したものである。

試験責任者： 雑賀 修  2010 年 7 月 21 日
雑賀 修
(株) 日曹分析センター 小田原事業所

Quality Assurance Statement

Study Number: NCAS10-066

Study Title: Acute toxicity test in *Oryzias latipes*

Quality Assurance inspections of the study referred above were conducted according to the appropriate GLP regulations and the standard operating procedures (SOPs) of the Quality Assurance Unit (QAU). The results of the inspections were reported to the study director and the facility management on the following dates.

| Items inspected | Dates (Month/Day/Year) | | |
|---|------------------------|----------------|------------|
| | Inspected | Reported to | |
| | | Study Director | Management |
| Protocol | 5/21/2010 | 5/21/2010 | 5/21/2010 |
| Experimental procedures | | | |
| • Acquisition and management of fish | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| • Preparation of the test solutions | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| • Exposure of fish to the test solutions | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| • Observation of fish | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| • Analytical sampling and treatment | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| • Analysis of the concentrations (LC/MS/MS) | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| • Preparation of the standard solutions | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| • Measurement of the NMR spectrum | 6/18, 22/2010 | 6/22/2010 | 6/22/2010 |
| Raw Data | 6/23-24/2010 | 6/24/2010 | 6/24/2010 |
| Draft Report | 6/23-24/2010 | 6/24/2010 | 6/24/2010 |
| Final Report | 7/21/2010 | 7/21/2010 | 7/21/2010 |

The QAU found that the study was performed according to the protocol and SOPs, the reported methods and procedures were actually used, and the results accurately reflect the recorded data.

QAU Manager: _____ (signature) _____ (seal) _____ (July 21, 2010)
 Ken Watabe
 Nisso Chemical Analysis Service Co., Ltd.

The original signature page of Quality Assurance Statement follows on page 5.

The original Quality Assurance Statement

The English translation of Quality Assurance Statement appears on page 4.

信頼性保証書

試験番号： NCAS 10-066

試験名：

ヒメダカに対する急性毒性試験

上記試験の信頼性保証の監査又は査察を適用 GLP および信頼性保証部門（QAU）の SOP に基づいて実施した。監査又は査察の結果は、以下の日付で試験責任者および運営管理者に報告した。

| 監査又は査察項目 | 日付（月/日/年） | | |
|-----------------|---------------|-----------|-----------|
| | 監査又は査察日 | 報告日 | |
| | | 試験責任者 | 運営管理者 |
| 試験計画書 | 5/21/2010 | 5/21/2010 | 5/21/2010 |
| 実験操作 | | | |
| ・魚の入手と管理 | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| ・試験溶液の調製 | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| ・試験溶液への暴露 | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| ・魚の観察 | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| ・分析試料の採取および処理 | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| ・濃度分析（LC/MS/MS） | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| ・標準溶液の調製 | 5/24/2010 | 5/24/2010 | 5/24/2010 |
| ・NMR スペクトル測定 | 6/18, 22/2010 | 6/22/2010 | 6/22/2010 |
| 生データ | 6/23-24/2010 | 6/24/2010 | 6/24/2010 |
| 報告書草案 | 6/23-24/2010 | 6/24/2010 | 6/24/2010 |
| 最終報告書 | 7/21/2010 | 7/21/2010 | 7/21/2010 |

QAU は、この試験が試験計画書および SOP に従って行われ、報告された方法や手段が実際に使われたものであり、結果は記録されたデータを正確に反映していることを確認した。

QAU 責任者

渡部 健

渡部 健

（株）日曹分析センター

2010 年 7 月 21 日

Study Information

Study No.: NCAS 10-066

Study Title: Acute toxicity test in *Oryzias latipes*

Report No.: NCAS 10-066

Sponsor:

Test Facility: Nisso Chemical Analysis Service Co., Ltd.
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TEL 81-465-42-5268, FAX 81-465-42-3586

Study Director: Osamu Saika

Experimenter: Tomoko Yoshimura, Noboru Fujikake, Satoko Ogawa, Chiaki Urabe (management of test organisms)
Tomoko Yoshimura (test solution preparation, observation and analysis of water parameter)
Shinpei Oonuki and Chiaki Urabe (analysis of concentration of test substance)
Shinpei Tsushima (Measurement of the NMR spectrum after the exposure termination)

Study Initiation Date: May 21, 2010

Experimental Start Date: May 24, 2010

Exposure Period: May 24, 2010–May 28, 2010

Experimental Completion Date: June 18, 2010

Study Completion Date: July 21, 2010

Test Guideline: Standard for the testing methods concerning new chemical substances. (Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, No.1121002, November 21, 2003; Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, No.2, November 13, 2003; Environmental Policy Bureau, Ministry of the Environment, No. 031121002) The latest amendment: November 20, 2006, Fish Acute Toxicity Test

Archiving: All the documents of this study will be retained in the archives of the test facility for

10 years after the completion of the study, but the place for storage after that will be decided on discussion with the sponsor. The test substance will be retained in the test facility at least for 10 years after the completion of the study, but only as long as the quality of the substance affords evaluation.

Deviation from the SOPs and the protocol: Trifluoroacetic acid used as a chemical shift standard for NMR measurement was over the expiry date specified in the SOP. However, it was considered that the deviation did not influence the results because trifluoroacetic acid did not decompose from the result of NMR measurement.

Circumstance / matter that may affect the reliability of the test results: None

Table of Contents

| | |
|--|----|
| Title Page | 1 |
| GLP Compliance Statement..... | 2 |
| The original GLP Compliance Statement | 3 |
| Quality Assurance Statement | 4 |
| The original Quality Assurance Statement | 5 |
| Study Information | 6 |
| Table of Contents | 8 |
| Summary | 10 |
| Introduction | 10 |
| Materials and Methods | 10 |
| 1. Test substance..... | 10 |
| 2. Verification of the test substance..... | 11 |
| 3. Verification of stability of the test substance..... | 11 |
| 4. Verification of solubility of test substance | 11 |
| 5. Reagents and apparatus | 11 |
| 6. Test organism | 12 |
| 7. Acclimatization | 13 |
| 8. Acute toxicity study | 13 |
| Results | 16 |
| 1. Validation of the stability of the test substance | 16 |
| 2. Validation of the analysis procedure of the test concentrations in test solutions | 16 |
| 3. The concentrations of the test substance in test solutions | 16 |
| 4. The concentrations of the test substance and mortality..... | 16 |
| 5. The median lethal concentration (LC ₅₀)..... | 16 |
| 6. Observations on the fish..... | 17 |
| 7. Observations on the test solutions..... | 17 |
| 8. Water temperature, pH and dissolved oxygen concentration in test solutions | 17 |
| Conclusions | 17 |
| Tables and Figures | 18 |
| Table 1 Result of validation of the analysis | 18 |
| Table 2 Concentration of test substance in test solution | 18 |
| Table 3 Number of deaths and mortality..... | 18 |
| Table 4 Observations of fish | 19 |
| Table 5 Observations of the test solution | 19 |
| Table 6 Water temperature in the test solution..... | 19 |
| Table 7 pH in the test solution | 20 |
| Table 8 Dissolved oxygen concentration in the test solution..... | 20 |

| | | |
|-----------|--|----|
| Figure 1 | NMR spectrum of supplied by Sponsor | 21 |
| Figure 2 | NMR spectrum of the test substance (before the exposure initiation)..... | 21 |
| Figure 3 | NMR spectrum of the test substance (after the exposure termination) | 22 |
| Figure 4 | Typical calibration curve | 23 |
| Figure 5 | Typical chromatogram of a standard solution (5.05 mg/L, at the start of the exposure) | 23 |
| Figure 6 | Typical chromatogram of a repeatability test sample | 24 |
| Figure 7 | Chromatogram of the control group at the start of the exposure | 24 |
| Figure 8 | Chromatogram of the exposure group (the 100 mg/L group) at the start of the exposure..... | 25 |
| Figure 9 | Chromatogram of the exposure group (the 100 mg/L group) after 48 hours of the exposure (before renewal) | 25 |
| Figure 10 | Chromatogram of the exposure group (the 100 mg/L group) after 48 hours of the exposure (after renewal) | 26 |
| Figure 11 | Chromatogram of the exposure group (the 100 mg/L group) at the end of the exposure.. | 26 |

Summary

The 96-hour acute toxicity study was performed to estimate the effect of the test substance, _____ to *Oryzias latipes* under semi-static (48-hour renewal cycle) and open system. A 24.0% aqueous solution of _____ was used as the test substance. In this report, the concentrations of _____ were expressed as _____, active ingredient, unless otherwise specially stated. On the basis of the analysis results of the concentrations of the test substance in the test solutions (LC/MS/MS), the variability was within $\pm 20\%$ of the nominal concentrations, therefore the median lethal concentrations (LC_{50}) were calculated on the basis of the nominal concentrations as the test concentrations during the exposure period. No mortality or abnormality was observed during the exposure period. The 24-, 48-, 72- and 96-hour LC_{50} are presented below.

| Exposure duration | LC_{50} (mg/L) |
|-------------------|------------------|
| 24 hours | > 100 |
| 48 hours | > 100 |
| 72 hours | > 100 |
| 96 hours | > 100 |

Introduction

This study was conducted in accordance with the following requirement to determine the median lethal concentrations (LC_{50}) by exposing the *Oryzias latipes* to the test solutions that contained _____ for 96 hours.

- Standard for the testing methods concerning new chemical substances. (Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, No.1121002, November 21, 2003; Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, No.2, November 13, 2003; Environmental Policy Bureau, Ministry of the Environment, No. 031121002) The latest amendment: November 20, 2006, Fish Acute Toxicity Test

Materials and Methods

1. Test substance

Name:

Abbreviated name:

Product name:

Chemical name:

Structure:

CAS No.:

Molecular formula:

Molecular weight:

Lot No.:

NCAS retrieval No.: STD-1150

Purity: Solid content: 24.0% aqueous solution (

Impurities: 0.85% (as against solid content), Cl ion; 35 ppm

In this report, the concentrations of the test substance were expressed as those of active ingredient, unless otherwise specially stated. was defined as the solid contents except water in this study.

Appearance:

Obtained from:

Acquisition volume: 420 g

Acquisition date: January 18, 2010

Expiration date: January 18, 2013

Storage conditions: Stored in a refrigerator with a polypropylene container

Water solubility: Soluble in water

Stability information: Stable in water

Summary of risk and harmfulness: Causes skin and eye irritation. Wear protective gloves, goggles and mask when it is used (from MSDS).

2. Verification of the test substance

The identity of the test substance was verified by comparing a ^{19}F -NMR spectrum which was measured before the exposure initiation with another spectrum which was supplied by the sponsor (Figure 1 and 2). This verification was conducted in another study, NCAS 10-022 "Determination of the Concentration of in Dosing Solutions".

3. Verification of stability of the test substance

The stability under storage condition was verified by confirming identity between two ^{19}F -NMR spectra which were measured before and after the exposure period.

4. Verification of solubility of test substance

Since the test substance was a 24% aqueous solution, its solubility was not confirmed.

5. Reagents and apparatus

Dechlorinated tap water: Odawara City tap water was dechlorinated by activated charcoal treatment and aerated sufficiently with an air pump before the test.

Quality of the water was confirmed to meet the standard criteria of 3rd-class fisheries water (Report No.: NCAS 09-064NG, provided from Chiba Pharmacist Association Inspection Center Foundation, Sample No.: D9C-8079, Sampling Day: December 8, 2009). The total hardness and the concentration of the residual chlorine were measured before the test and it was confirmed that the total hardness as CaCO_3 was 60 mg/L, within the prescribed range (total hardness as CaCO_3 range from 40 to 100 mg/L), and the concentration of the residual chlorine was <0.01 mg/L.

| | |
|--|---|
| Ion exchange water: | Tap water was distilled with a Barnstead water distilling apparatus (WDA-15S, Isuzu Seisakusho Co., Ltd.) and then purified with an ultrapure water system (LV-08, Toray Co., Ltd.) |
| Acetonitrile: | HPLC grade (Wako Pure Chemical Industries, Ltd.) |
| Formic acid: | Special grade (Wako Pure Chemical Industries, Ltd.) |
| Trifluoroacetic acid: | HPLC grade (Wako Pure Chemical Industries, Ltd.) |
| Hardness measurement kit: | WAD-TH (Kyoritsu Chemical-check Lab., Corp) |
| Balances: | AG285, AX205 (Mettler Toledo), BX-3200H (Shimadzu Co.) |
| Thermostat water bath: | NTT-1200 (Tokyo Rikakikai Co., Ltd.), |
| Thermo recorder: | SK-L200T (Sato KEIRYOKI MTG Co., Ltd.) |
| Dissolved oxygen meter: | OM-51 (HORIBA, Ltd.) |
| pH meter: | D-51 (HORIBA, Ltd.) |
| Thermometer: | Standard thermometer; measuring temperature range from 0 to 50°C, minimum scale 0.1°C, (Ando Keiki Co., Ltd.) |
| Residual chloride meter: | Digital residual chloride tester DCT-300 (Takumina Co., Ltd.) |
| Centrifuge: | KN-70 (Kubota Co.) |
| Micropipette: | Eppendorf Research V (Eppendorf Co., Ltd.) |
| Centrifuge tube: | 10-mL glass test tube with ground-in stopper |
| Nuclear chromatograph resonance apparatus: | JNM-ECA500 (JEOL Ltd.) |
| LC/MS/MS: | Acquity / Quattro micro API (Waters Corp.) |

6. Test organism

| | |
|----------------------------------|--|
| Species: | <i>Oryzias latipes</i> |
| Obtained from: | Kitamura Fish Farm (obtained on May 11, 2010) |
| Birth month and year: | October 2009 |
| Average total length and weight: | 2.4 cm (2.3–2.5 cm), 0.10 g (0.08–0.12 g) |
| | The average value and range of 10 fish randomly selected at the exposure initiation. |
| Sensitivity: | The background data of the 96-hour LC_{50} value of the reference substance (copper (II) sulfate) in our laboratory; 0.59–2.0 mg/L (August 2008–February 2010, n=5) |

7. Acclimatization

Oryzias latipes were obtained on May 11, 2010 and treated with a medicated bath of ELEVAGE (Japan Pet Design Co., Ltd) and salt for 6 hours on the same day. After 3 days of the holding, the fish were acclimatized for 10 days (May 14–24, 2010) under the conditions described below. Mortality of 4.6% occurred for 7 days before the exposure initiation.

| | |
|---------------------------------|---|
| Holding water: | Dechlorinated tap water |
| Holding chamber: | 100-L glass chamber |
| Water temperature: | 22.7–23.4 °C |
| Dissolved oxygen concentration: | 95–99% (of saturation) |
| pH: | 7.3–7.5 |
| Lighting: | fluorescent light, 16 hour light / 8 hours dark |
| Feed: | MEDAKANOESA (Tetra Japan Co., Ltd) |
| Feeding volume: | Foods were given at a rate of 1.5–1.8% of fish weight once a day. No feeds were supplied for 24 hours before the exposure initiation. |
| Holding system: | Flow-through |
| Test room: | A6 |

8. Acute toxicity study

8.1 Test conditions

| | |
|---------------------------------|--|
| Exposure system: | Semi-static (48-hour renewal cycle, open system) |
| Exposure duration: | 96 hours |
| Test chamber: | 3-L glass beaker |
| Volume of test solutions: | 3 L / test chamber |
| Replicates: | 1 test chamber / group |
| Number of test organism: | 7 fish / group |
| Dilution water: | Dechlorinated tap water (the concentration of the residual chlorine was <0.01 mg/L, the total hardness was 60 mg/L (as CaCO ₃)). |
| Aeration: | Not conducted |
| Test temperature: | 22.8–22.9°C (water temperature in the water bath during the exposure period; 23.1–23.3°C) |
| pH: | 7.4–8.0 |
| Dissolved oxygen concentration: | 77–95% of saturation |
| Lightning: | Fluorescent light, 16 hour light / 8 hours dark |
| Feeding: | No feed was supplied for 48 hours before the exposure. |
| Test room: | A8 |

8.2 Selection of the test concentrations and exposure systems

Based on the results of a 96-hour semi-static range-finding test (Report No.: NCAS 10-063NG), no mortality or abnormality was observed at the nominal concentration of 100 mg/L that was the limit concentration as active ingredient. The nominal concentrations were maintained during the 48-hour

static period. Therefore, the definitive test (semi-static system for 96 hours, 48-hour renewal cycle) was conducted at the highest concentration of 100 mg/L.

8.3 Preparation of test solutions

Test solutions were prepared at the start and after 48 hours of the exposure. An amount of test substance (300 mg: 1249.8 mg as aqueous solution) was weighed and transferred to a 1000-mL volumetric flask and dissolved in dechlorinated tap water. This solution was transferred into the test chamber and then added 2 L dechlorinated water to prepare the exposure solution.

8.4 Analysis of test concentrations

8.4.1 LC/MS/MS conditions

| | |
|---------------------|---|
| Apparatus: | Acquity / Quattro micro API (Waters) |
| Column: | Acquity UPLC BEH C18, 2.1 mm i.d. × 50 mm, particle diameter: 1.7 μm (Waters) |
| Mobile Phase: | Acetonitrile + 0.1% formic acid aqueous solution (v/v) = 50 + 50 (v/v) |
| Flow rate: | 0.3 mL/min |
| Column temperature: | 40°C |
| Injection volume: | 5 μL |
| Ionization mode: | ESI, Negative |
| Monitoring ion: | 180.33 > 96.90 (quantification), 180.33 > 77.84 (confirmation) |

8.4.2 Preparation of standard solutions and calibration curve

An amount (5.05 mg, as) of the test substance was correctly weighed and transferred into a 50-mL volumetric flask and dissolved in 50% acetonitrile aqueous solution to prepare a 101 mg/L standard stock solution. This standard stock solution was then further diluted with the same solvent to prepare 1.01, 2.02, 3.03, 4.04 and 5.05 mg/L standard solutions. These standard solutions were analyzed by using the LC/MC/MC conditions described in section 8.4.1. Calibration curves were prepared by plotting the peak areas vs. the concentrations of and the linear regression equations and correlation coefficients (r) were calculated by using a computer program of LC/MS/MS (Mass Lynx v 4.1). The accuracy (%) for each calibration solution was calculated by inverse regression method. The weighting of 1/x was carried out. The standard solutions used to construct calibration curves were prepared before use.

8.4.3 Analysis of the concentration of the test substance in test solutions

The test solutions were analyzed at the exposure initiation, before and after the 48-hour renewal, and the exposure termination. Samples were mixed with acetonitrile at the rate of 1:1(v/v) and centrifuged (3000 rpm, 10 min). The supernatant fluids obtained were diluted with 50% acetonitrile aqueous solution to be appropriate concentration which was approximated in the middle of the range of the calibration curve, and then analyzed by using the LC/MS/MS conditions as described in section 8.4.1.

The samples from the control group were also mixed with acetonitrile at the rate of 1:1(v/v) and

centrifuged (3000 rpm, 10 min) and then analyzed by the same procedures to verify that any interfering peak did not appear at the retention time of the test substance on the chromatogram.

8.5 Validation study of the analysis of the test substance concentration in test solutions

8.5.1 Validation of the calibration curve

The accuracy was calculated by finding the regression equation and correlation coefficient (r) of the calibration curves, and then determining the quantitative value with inverse estimation method.

The criteria for the calibration curve were described below.

1. The correlation coefficient is more than 0.990.
2. The accuracy for the lowest concentration solution is within $\pm 20\%$, and that for other solutions is within $\pm 15\%$.

8.5.2 Validation of the repeatability

Analytical method was validated by a repeatability test without any recovery tests, because extract operation was not conducted. The concentrations of the test substance in the exposure group were calculated by the same procedure as described in section 8.4.3 ($n=3$). The analytical method was determined to be validated if the coefficient of variations of the measured test concentrations ($n=3$) was within $\pm 10\%$.

8.6 Exposure procedure

At the start of the exposure, the weights and total lengths of 10 fish from the acclimated fish in this study were measured, and the total lengths were confirmed to be 2.2 ± 1.0 cm.

After measurement of water temperature, dissolved oxygen concentration and pH in each test chamber, 7 fish were added to each chamber. Water temperature, dissolved oxygen and pH were measured at the exposure initiation, before and after the 48-hour renewal and at the end of the exposure. No feed was supplied during the exposure period. Temperature in the thermostat water bath, in which the test chambers were placed, was continuously recorded during the exposure period with a thermo recorder to monitor the change of the water temperature. Fish were observed and recorded just after exposure initiation, and after 3, 6, 24, 48, 72 and 96 hours of the exposure initiation. The conditions of the test solutions were observed and recorded just after the exposure initiation, and after 24, 48, 72 and 96 hours of the exposure.

The test solutions were prepared as described in section 8.3 after 48 hours of the exposure initiation and surviving fish were transferred to them.

8.7 Calculation of the median lethal concentration (LC_{50})

The ranges of LC_{50} were determined from the number of dead fish in each test group after 24, 48, 72 and 96 hours of the exposure.

Results

1. Validation of the stability of the test substance

Since the spectrum which was measured before the exposure initiation (Figure 2) was corresponded to that was measured after the exposure termination (Figure 3), it was determined that the test substance was stable during the storage period.

2. Validation of the analysis procedure of the test concentrations in test solutions

2.1 Validation of the calibration curve

A typical calibration curve is shown in Figure 4 and a typical chromatogram of the standard solution (5.05 mg/L, at the start of the exposure) is shown in Figure 5.

The coefficients of correlation (r) were more than 0.990 at the exposure initiation, after 48 hours and end of the exposure. The all variations of the accuracy of the lowest standard samples were within $\pm 20\%$ and those of other standard samples were within $\pm 15\%$. Based on the results, the linearity of the calibration curves were determined as acceptable.

2.2 Validation of the repeatability

The results of the repeatability test are shown in Table 1. A typical chromatogram of the repeatability test (nominal concentration: 100 mg/L) is shown in Figure 6. A chromatogram of the control group is shown in Figure 7.

The Coefficients of variation ($n=3$) of the measured concentrations of the test substance were within 10%. On the chromatogram of the control group, no interfering peak appeared at the retention time of the test substance. On the basis of these results, the analytical method of the test substance concentration in the test solution was determined to be valid in this study.

3. The concentrations of the test substance in test solutions

The analytical results of the test substance concentrations in the test solutions are presented in Table 2 and chromatograms of the exposure solution at the exposure initiation, after 48 hours (before and after renewal) of the exposure and at the exposure termination are shown in Figure 8–11 respectively.

The test substance concentrations in the test solutions ranged from 99.0 to 103% of the nominal during the exposure period. Since the variability of the measured concentrations was within $\pm 20\%$ of the nominal, the nominal concentrations were considered as the test concentrations.

4. The concentrations of the test substance and mortality

The number and percentage of deaths in each test group are shown in Table 3.

No mortality occurred both in control and the 100 mg/L groups at any hours of exposure duration.

5. The median lethal concentration (LC_{50})

The 24-, 48-, 72- and 96-hour LC_{50} are determined to be >100 mg/L since there were no dead fish at any hours of exposure duration.

6. Observations on the fish

The results of the observation were shown in Table 4.

No abnormality was observed in the control or exposure groups at any hours of the exposure duration.

7. Observations on the test solutions

The observation results of the test solutions were shown in Table 5. Throughout the exposure period, no turbidity or precipitate was observed and the color was clear and transparent in any groups.

8. Water temperature, pH and dissolved oxygen concentration in test solutions

The measurements of temperature, pH and dissolved oxygen concentration in the test solutions are presented in Table 6, 7 and 8 respectively. The water temperatures ranged from 22.8 to 22.9 °C and the pH ranged from 7.4 to 8.0 during the exposure period. The dissolved oxygen concentrations ranged from 77 to 94% which were maintained $\geq 60\%$ of saturation.

Conclusions

The 96-hour acute toxicity study under semi-static conditions (48-hour renewal cycle and open system) was performed by exposing *Oryzias latipes* to It is concluded
that the 96-hour median lethal concentration (LC_{50}) is >100 mg/L.

Tables and Figures

Table 1 Result of validation of the analysis

| Nominal concentration (mg/L) | Measured concentration (mg/L) | | | Mean measured concentration (mg/L) | Coefficient of variation (%) |
|------------------------------|-------------------------------|-----|-----|------------------------------------|------------------------------|
| 100 | 102 | 105 | 105 | 104 | 1.67 |

Table 2 Concentration of test substance in test solution

| Nominal concentration (mg/L) | The measured concentration of test substance at each exposure duration (mg/L) | | | |
|------------------------------|---|---------------------------|--------------------------|-------------|
| | 0 hour | 48 hours (before renewal) | 48 hours (after renewal) | 96 hours |
| Control | N.D. | N.D. | N.D. | N.D. |
| 100 | 103 (103) | 101 (101) | 100 (100) | 99.0 (99.0) |

(): Percent of nominal concentration (%)

N.D.: Not detected

Table 3 Number of deaths and mortality

| Nominal concentration (mg/L) | Cumulative number of deaths (Mortality (%)) | | | | | |
|------------------------------|---|---------|----------|----------|----------|----------|
| | 3 hours | 6 hours | 24 hours | 48 hours | 72 hours | 96 hours |
| Control | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 100 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

Table 4 Observations of fish

| Nominal concentration (mg/L) | Observations at each exposure duration | | | | | | | |
|------------------------------|--|---------|---------|----------|------------------------|------------------------|----------|----------|
| | 0 hour | 3 hours | 6 hours | 24 hours | 48 hours* ¹ | 48 hours* ² | 72 hours | 96 hours |
| Control | N-7 | N-7 | N-7 | N-7 | N-7 | N-7 | N-7 | N-7 |
| 100 | N-7 | N-7 | N-7 | N-7 | N-7 | N-7 | N-7 | N-7 |

N: Normal

*¹: Before renewal*²: After renewal

Table 5 Observations of the test solution

| Nominal concentration (mg/L) | Observations at each exposure duration | | | | | |
|------------------------------|--|-----------------------|------------------------|------------------------|-----------------------|-----------------------|
| | 0 hour | 24 hours | 48 hours* ¹ | 48 hours* ² | 72 hours | 96 hours |
| Control | Clear and transparent | Clear and transparent | Clear and transparent | Clear and transparent | Clear and transparent | Clear and transparent |
| 100 | Clear and transparent | Clear and transparent | Clear and transparent | Clear and transparent | Clear and transparent | Clear and transparent |

*¹: Before renewal*²: After renewal

Table 6 Water temperature in the test solution

| Nominal concentration (mg/L) | Water temperature at each exposure duration (°C) | | | |
|------------------------------|--|---------------------------|--------------------------|----------|
| | 0 hour | 48 hours (before renewal) | 48 hours (after renewal) | 96 hours |
| Control | 22.8 | 22.9 | 22.9 | 22.9 |
| 100 | 22.8 | 22.9 | 22.9 | 22.9 |

Table 7 pH in the test solution

| Nominal concentration (mg/L) | pH at each exposure duration | | | |
|------------------------------|------------------------------|---------------------------|--------------------------|----------|
| | 0 hour | 48 hours (before renewal) | 48 hours (after renewal) | 96 hours |
| Control | 7.7 | 7.5 | 7.9 | 7.5 |
| 100 | 7.8 | 7.7 | 8.0 | 7.4 |

Table 8 Dissolved oxygen concentration in the test solutions

| Nominal concentration (mg/L) | Dissolved oxygen concentration at each exposure duration (%) | | | |
|------------------------------|--|---------------------------|--------------------------|----------|
| | 0 hour | 48 hours (before renewal) | 48 hours (after renewal) | 96 hours |
| Control | 94 (7.9) | 77 (6.5) | 93 (7.8) | 83 (7.0) |
| 100 | 94 (7.9) | 79 (6.6) | 95 (8.0) | 77 (6.5) |

Figures in parentheses indicate the concentration.

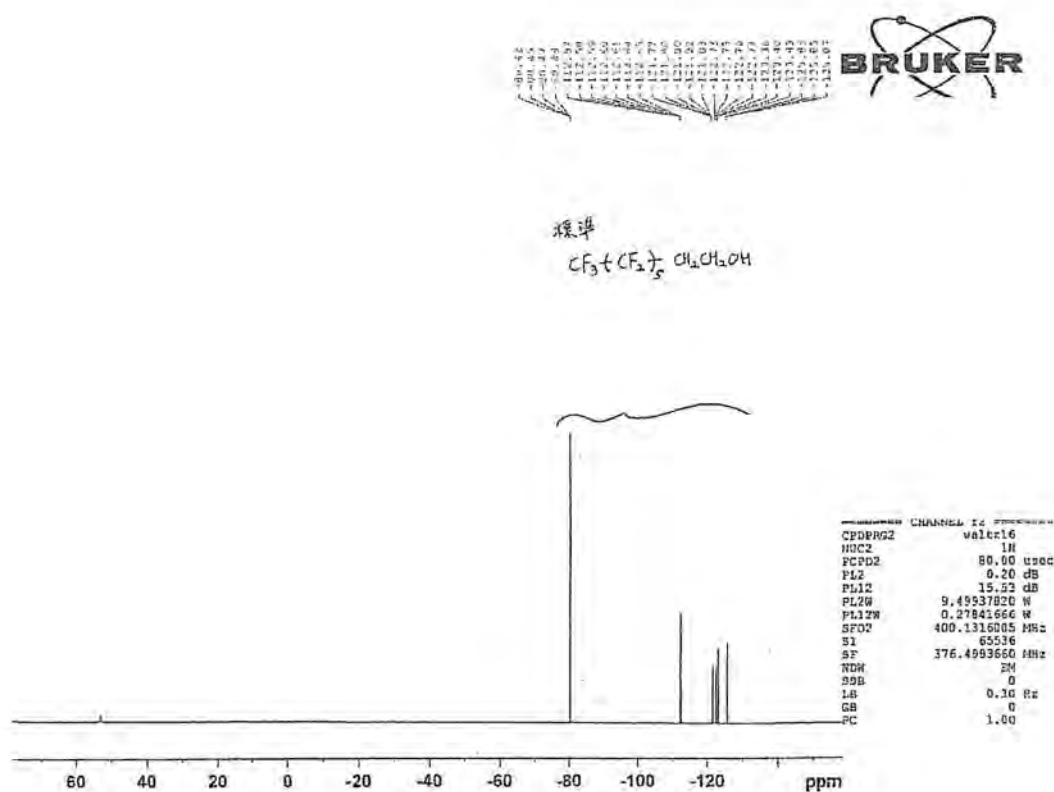



Figure 1 NMR spectrum of  supplied by Sponsor

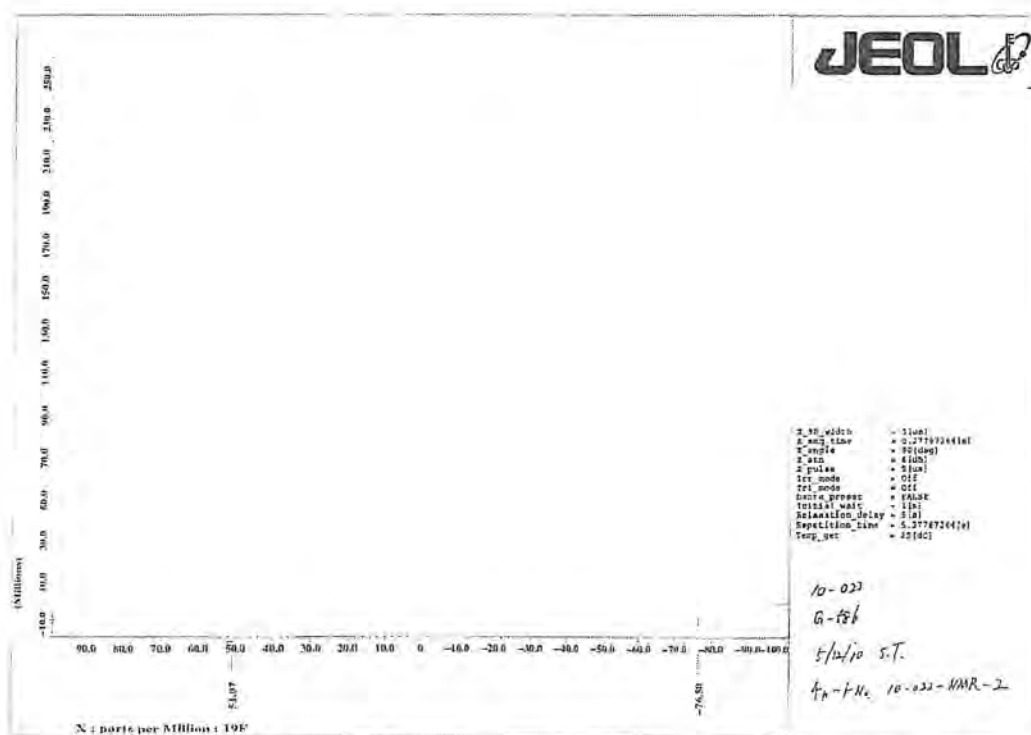


Figure 2 NMR spectrum of the test substance (before the exposure initiation)

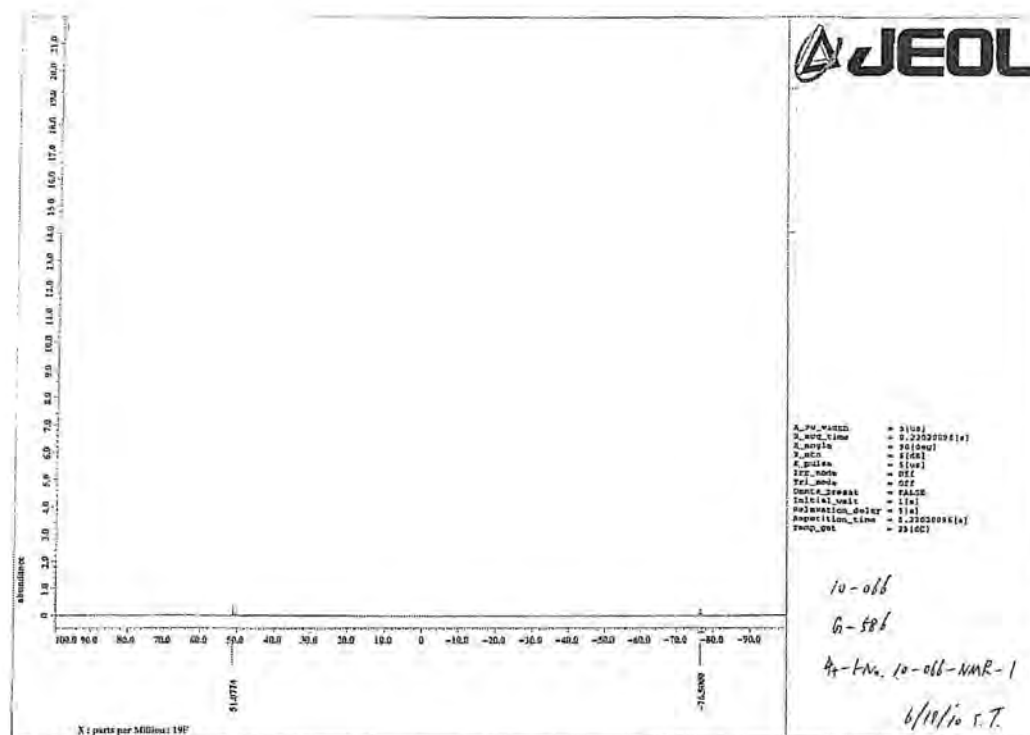


Figure 3 NMR spectrum of the test substance (after the exposure termination)

| Concentration (mg/L) | Peak area | Quantitative value (mg/L) | Accuracy (%) |
|-------------------------|-----------|---------------------------------|-----------------|
| 1.01 | 2006 | 0.956 | 94.7 |
| 2.02 | 3728 | 2.13 | 106 |
| 3.03 | 5151 | 3.11 | 103 |
| 4.04 | 6471 | 4.01 | 99.3 |
| 5.05 | 7830 | 4.94 | 97.8 |

Method: C:\MassLynx\ (MethDB)
Calibration: 24 May 2010 16:38:14

11 Apr 2010 18:23:08

定量用

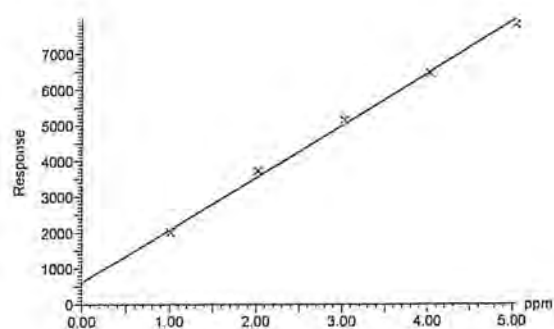


Figure 4 Typical calibration curve

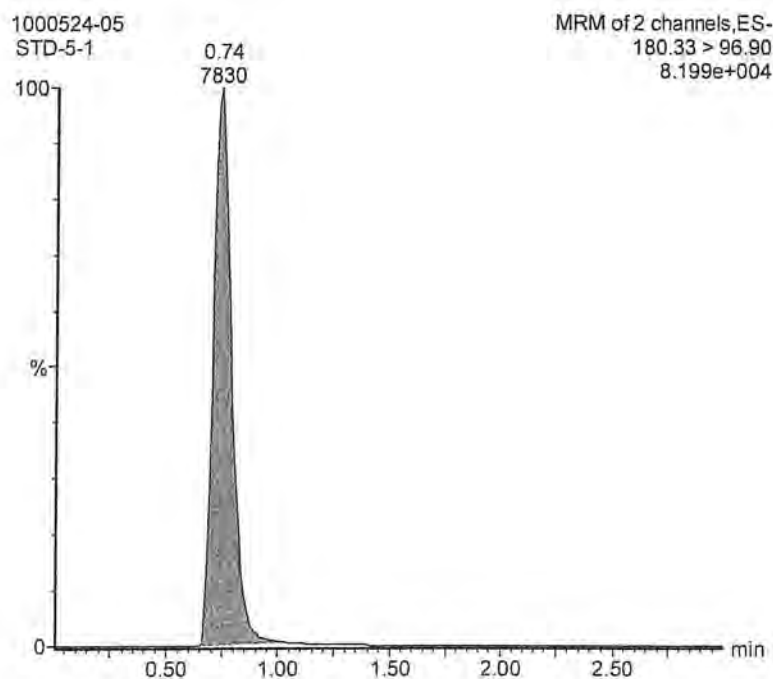


Figure 5 Typical chromatogram of a standard solution
(5.05 mg/L, at the start of the exposure)

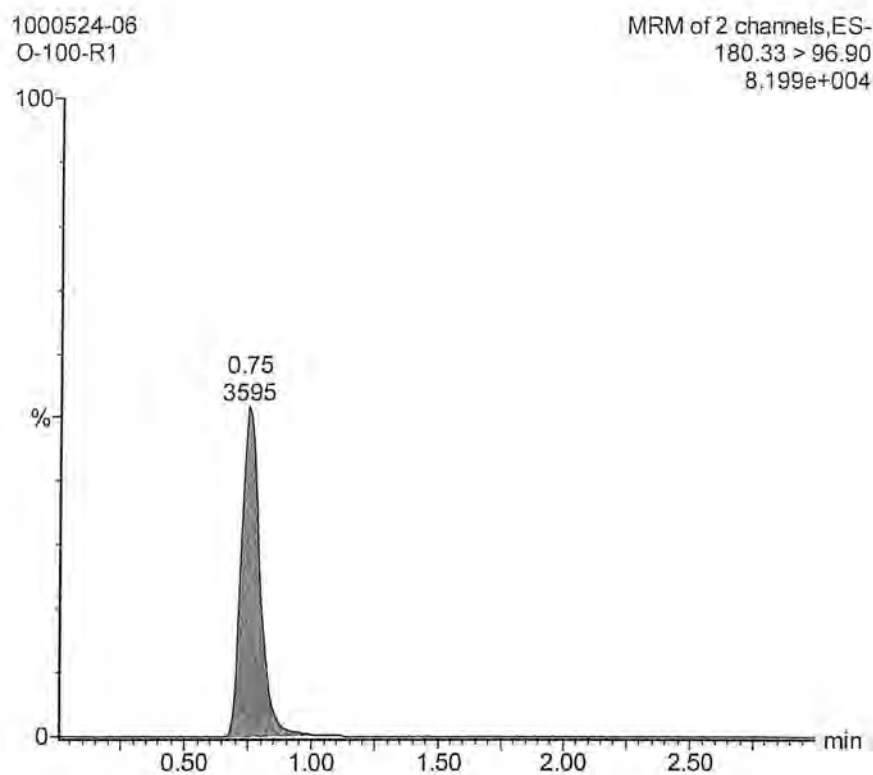


Figure 6 Typical chromatogram of a repeatability test sample

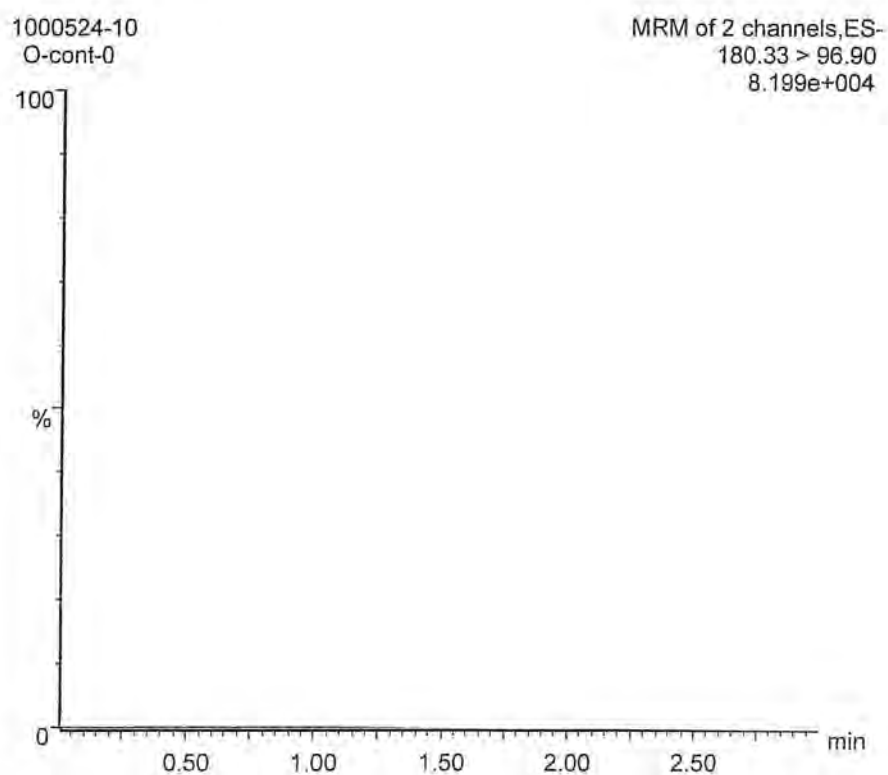


Figure 7 Chromatogram of the control group at the start of the exposure

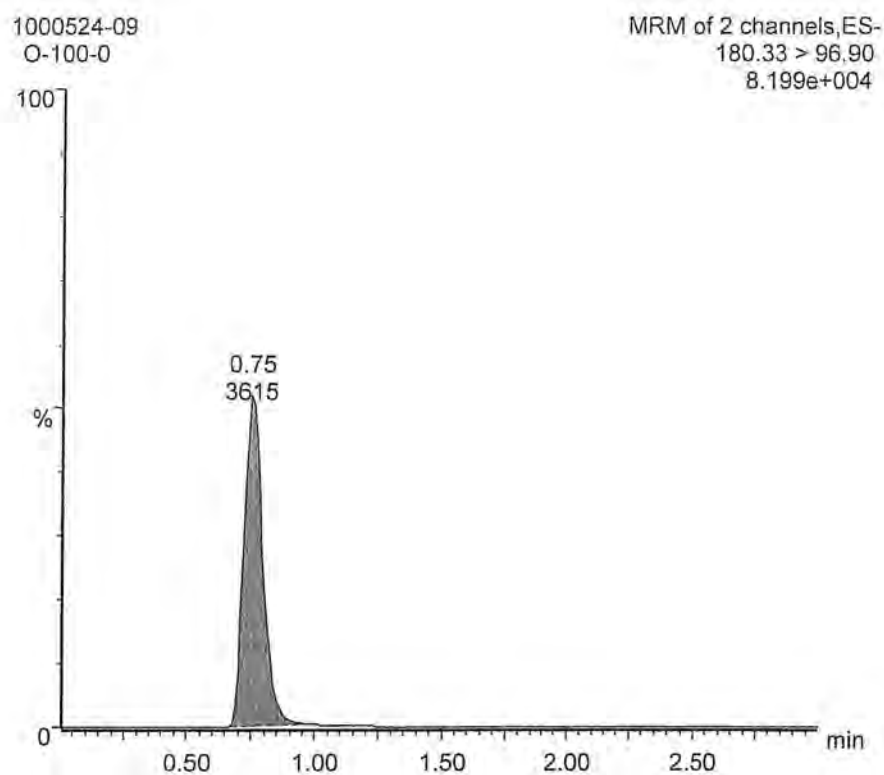


Figure 8 Chromatogram of the exposure group (the 100 mg/L group) at the start of the exposure

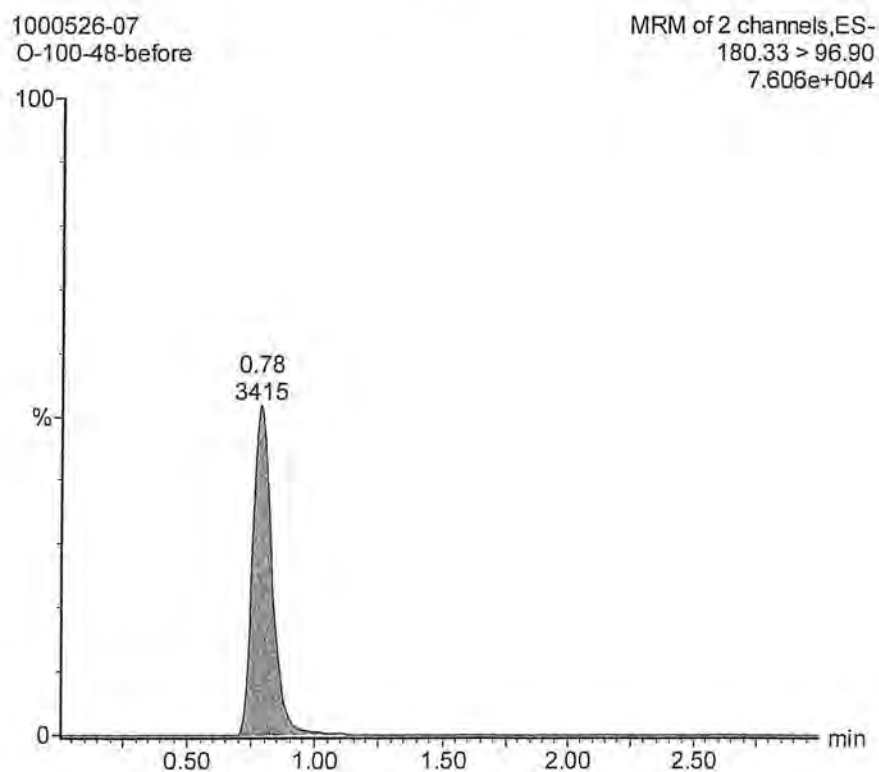


Figure 9 Chromatogram of the exposure group (the 100 mg/L group) after 48 hours of the exposure (before renewal)

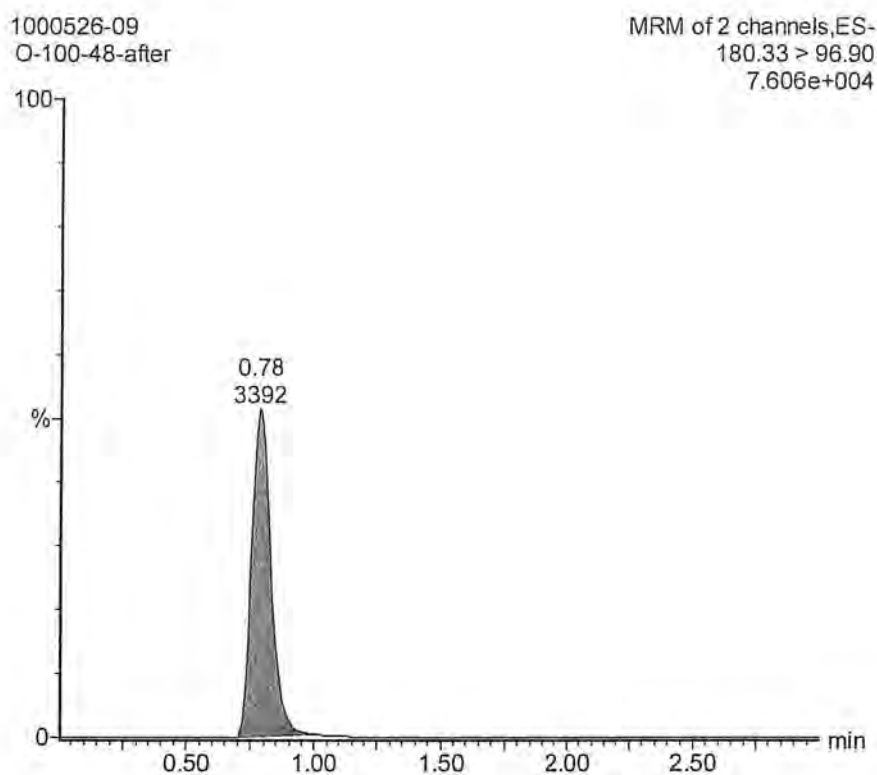


Figure 10 Chromatogram of the exposure group (the 100 mg/L group)
after 48 hours of the exposure (after renewal)

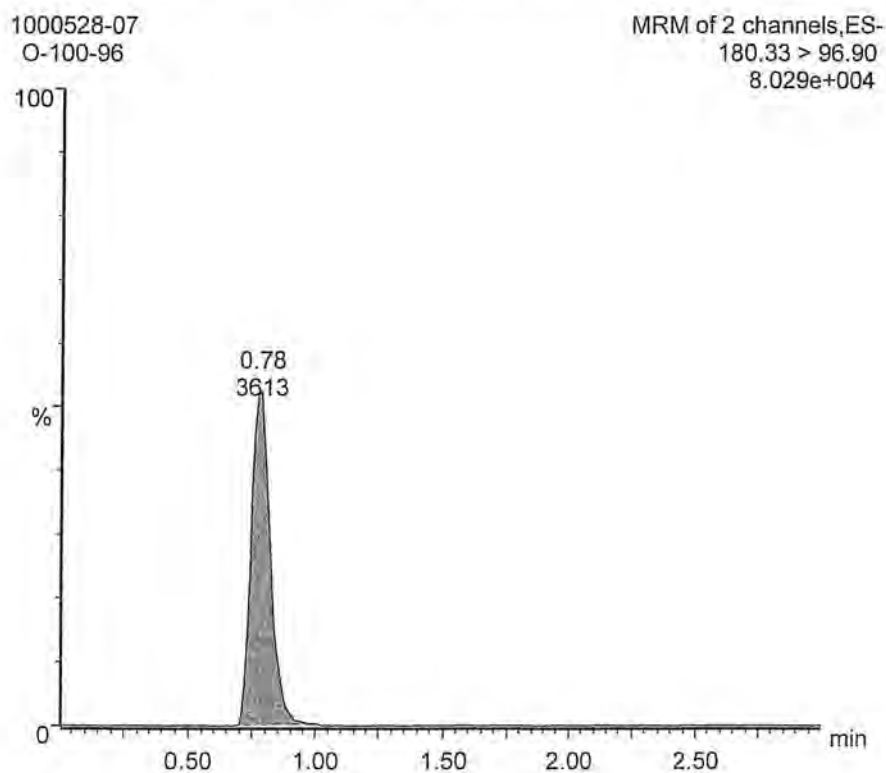


Figure 11 Chromatogram of the exposure group (the 100 mg/L group)
at the end of the exposure

Authenticity of Translation

I declare that the original Japanese final report (Report No. NCAS 10-066) is translated into English consistently.

Translated by :



Osamu Saika

Nisso Chemical Analysis Service Co., Ltd.

Odawara Laboratory

5 June, 2012